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Antibody Responses of Chimpanzees Immunized with Synthetic Peptides Corresponding to Full-Length V3 Hypervariable Loops of HIV-1 Envelope Glycoproteins

A. ROBERT NEURATH,¹ SHIBO JIANG,¹ NATHAN STRICK,¹ HANNO KOLBE,²
MARIE-PAULE KIENY,² ELIZABETH MUCHMORE,⁴ and MARC GIRARD³

ABSTRACT

Immunization of primates or humans with human immunodeficiency virus type 1 (HIV-1) glycoproteins usually elicited moderate immune responses to the principal neutralizing determinant (PND) located within the V3 hypervariable loop of gp120. Since an antibody response to the PND appears to be protective, experiments were carried out to determine the responsiveness of chimpanzees to immunization with synthetic peptides corresponding to the full-length V3 loop. Seven chimpanzees (4 preimmunized with gp160, 2 preimmunized with HIV-1 antigens unrelated to gp160, and 1 unimmunized) were vaccinated with a mixture of full-length V3 loop peptides from 21 distinct HIV-1 isolates (clones) either in unconjugated form or linked to carrier proteins from HIV-1 *nef* and *gag* P18, respectively. Six chimpanzees developed high levels of antibodies to the peptides (dilution endpoints 1: > 25,000), and 5 had high levels of antibodies to gp120 from HIV-1_{IIIB} (endpoint titers 1: > 500,000). Chimpanzees immunized with peptide-carrier conjugates (4) had antibodies to the carrier proteins *nef* and *gag* P18, respectively (endpoint titers 1: ≥ 35,000). Virus-neutralizing (VN) antibodies were detected in sera of 5 of 7 chimpanzees, but were present at titers of 1: ≥ 400 only in sera of 2 chimpanzees. One of these was challenged with HIV-1 and was protected against infection, as reported elsewhere. The antibodies were primarily specific for the HIV-1 isolate used for primary immunization before boosting with peptides. The relatively low dilution endpoints of VN antibodies as compared with endpoints determined by site-specific immunoassays probably can be ascribed to imperfect mimicry of conformational epitopes by synthetic peptides. Nevertheless, sequential or simultaneous immunization with recombinant envelope glycoproteins of HIV-1 and selected synthetic peptides offers an approach for eliciting protective immunity against HIV-1.

INTRODUCTION

VIRUS-NEUTRALIZING ANTIBODIES expected to be protective against the human immunodeficiency virus type 1 (HIV-1) are primarily directed against epitopes on the hypervariable loop from the V3 region of the HIV-1 envelope glycoprotein gp120.¹⁻¹³ Virus-neutralizing antibodies recognize predominantly the central portion of the V3 loop. Because of structural constraints on the variability of this segment of the V3 loop, it was suggested that peptide immunogens corresponding to contiguous sequences of the principal neutralizing determinant (PND) would induce antibodies neutralizing a majority of HIV-1

isolates.¹⁴⁻¹⁶ The combination of several peptides from the V3 loop of distinct HIV-1 isolates is also expected to result in production of virus-neutralizing antibodies with broad specificity.^{17,18}

Antibodies specific for the V3 loop were virus neutralizing not only in in vitro assays but they also abrogated the infectivity of HIV-1 for chimpanzees.¹⁹ This suggested that immunogens eliciting antibodies to the V3 loop may also confer protection against HIV-1 infection. Recent experimental findings confirm this expectation.^{20,21} Earlier studies indicated that chimpanzees immunized with gp120 purified by immunoaffinity chromatography from HIV-1-infected cells developed antibodies detect-

¹Lindsley F. Kimball Research Institute of the New York Blood Center, 310 East 67th Street, New York, NY 10021.

²Transgene, 11, rue de Molsheim, 6700 Strasbourg, France.

³Institut Pasteur, Direction des Applications de la Recherche, 28 rue du Docteur Roux, 75724 Paris, France.

⁴Laboratory for Experimental Medicine and Surgery in Primates, New York University Medical Center, 550 First Avenue, New York, NY 10016.

of goat antibodies raised by immunization with HIV-1 gp120 BH10 and SF2, respectively, on the reaction of rabbit antibodies to the respective V3 hypervariable loop peptides with wells coated with either homologous gp120 glycoproteins or with the corresponding V3 peptides was studied. Dilutions of the respective goat and anti-gp120 antisera in TS-BG were incubated with the wells for 30 min at 20°C. Subsequently, rabbit antisera to synthetic peptides corresponding to the V3 loops of HIV-1 BH10 and SF2, respectively, were added to a final dilution of 1:1,000. After incubation overnight at 25°C, the wells were washed with TS and the quantity of attached rabbit IgG was determined from the subsequent attachment of ¹²⁵I-labeled second antibodies as described before.⁹

To determine whether or not segments of the gp120 sequence nonadjacent within the primary amino acid sequence to the V3 hypervariable loop may occur in the proximity of the V3 hypervariable loop due to folding within the three-dimensional structure of the HIV-1 envelope, the inhibitory effect of MAb (NEA-9305; Du Pont) (final dilution 1:500) and of goat antiserum anti-SP10 (final dilution 1:20), both specific for the V3 hypervariable loop, on the attachment of rabbit antisera to peptides from the entire gp160 sequence⁹ was studied using wells coated with recombinant gp160 (500 ng/ml; MicroGene-Sys, Inc., West Haven, CT). The wells were incubated with the MAb and the goat antiserum, respectively, for 30 min at 20°C. Subsequently, dilutions of rabbit antipeptide antisera were added to the wells. All antisera were diluted in 1% fetal bovine serum, 1% goat serum, pH 8. After incubation overnight at 20°C, the attachment of rabbit IgG to the wells was determined from the subsequent adsorption of ¹²⁵I-labeled antirabbit IgG as described above. In addition to the antipeptide antisera described before,⁹ two additional antisera to the peptide (113-142) from HIV-1 BH10 gp120 (sequence in one-letter code: E-D-I-I-S-L-W-D-Q-S-L-K-P-C-V-K-L-T-P-L-C-V-S-L-K-C-T-D-L-K) were tested. The peptide was synthesized in two forms: the cysteine (C) residue at position 126 [(113-142)_{Aba126}], and the C at position 133 [(113-142)_{Aba133}], were replaced by aminobutyric acid (Aba) residues. These peptides were recognized by both human and rabbit anti-HIV-1 antibodies and elicited in rabbits high levels of antibodies recognizing recombinant gp160 (gp120) (endpoint titers 1:100,000 to 1:400,000) (own unpublished data).

To study the effect of disulfide bond formation between N- and C-terminal cysteine residues within the V3 hypervariable loop peptide from HIV-1 BH10 on its antigenicity, the peptide was reduced with 1% 2-mercaptoethanol for 1 h at 20°C followed by treatment with iodoacetamide (final concentration 30 mg/ml) for 1 h at 20°C, followed by dialysis against PBS. The antigenicity of the original and the reduced-alkylated peptides were compared. Wells of polystyrene plates were coated with MAb NEA9305 raised against the synthetic peptide R-I-Q-R-G-P-G-R-A-F-V-T-I-G-K corresponding to residues (315-329) of the V3 loop of HIV-1 BH10, or with MAb NEA9284. Horseradish peroxidase-labeled HIV-1 gp120 IIIB (final dilution 1:1,000 in 1% BSA in TS; American Bio-Technologies, Inc.) was added to the wells which were subsequently incubated for 2 h at 20°C in the absence (= positive control) or the presence of graded quantities of untreated and reduced-alkylated V3 peptide, respectively. The attachment of enzyme-labeled gp120 was determined by measurement of peroxidase activity as described

above for ELISA tests. The inhibitory effect of the peptides was calculated from the formula:

$$\% \text{ Inhibition} = \frac{\text{OD}_{450} \text{ in absence of peptides} - \text{OD}_{450} \text{ in the presence of peptides}}{\text{OD}_{450} \text{ in the absence of peptides}} \times 100,$$

where OD₄₅₀ = optical density at 450 nm. All values were corrected for the attachment of enzyme-labeled gp120 to control wells coated with BSA and gelatin.

Virus neutralization assays

Chimpanzee sera obtained before and after immunization with V3 loop peptides were tested. The titer of virus-neutralizing antibodies was determined by two distinct methods, based on the inhibition of synthesis of the core protein P24 and on protection by antibodies of cells against the cytopathic effect of HIV-1 (colorimetric method), respectively.

Briefly, the antisera were filtered through 0.2 µm Centrex cellulose acetate microfilters (Schleicher & Schuell, Inc., Keene, NH) and serially diluted in RPMI-1640 medium without phenol red (GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS). Aliquots of the filtered samples were added to wells of 96 well plates and mixed with an equal volume of diluted HIV-1_{IIIB}, RF, and MN, respectively [multiplicity of infection (MOI) = 0.0045]. After incubation for 1 h at 37°C, 25 µl of polybrene (1 µg/ml) treated MT-2 cells (5000 cells/well) were added. The mixture was incubated for 1 h at 37°C and the volume was adjusted with RPMI-1640 medium with 10% FCS to 200 µl. On the fourth and sixth day after incubation at 37°C, 100 µl of culture supernatants were collected from each well and equal volumes of fresh medium were added to the wells. The supernatants were assayed for P24 using a kit from Coulter Immunology (Hialeah, FL). On the sixth day, an indicator XTT tetrazolium dye (1 mg/ml; 50 µl/well; PolySciences, Inc., Warrington, PA) was added to the cells. After 4 h, intracellular formazan was determined colorimetrically at 450 nm.

RESULTS

Antibody responses of chimpanzees immunized with V3 hypervariable loop peptides from 21 distinct HIV isolates (clones)

Seven chimpanzees were immunized either with a mixture of unconjugated V3 loop peptides from 21 distinct HIV-1 isolates (clones) (chimpanzees #499, #151, #395; Fig. 1), with the same peptides covalently linked to recombinant *nef* protein (chimpanzees #529, #493, and #495), or sequentially with unconjugated peptides followed by peptides linked to recombinant *gag* P18 (chimpanzee #531). All chimpanzees except #531 were preimmunized with different HIV-1 antigens before receiving the peptide immunogens. Chimpanzee #499 was preimmunized with recombinant gp160 and *gag* P18 using formulations of these immunogens described before.²¹ Chimpanzees #151, #395, and #493 were preimmunized with a

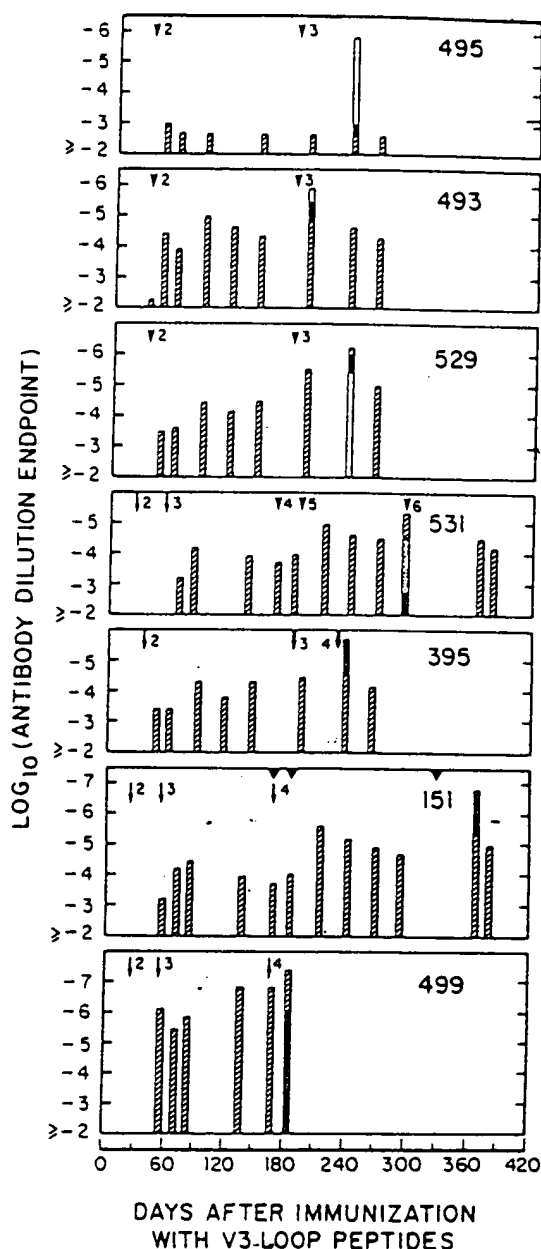


FIG. 1. Antibody responses of chimpanzees immunized with preparations of V3 hypervariable loop peptides from 21 distinct HIV-1 isolates (clones). Chimpanzees 499, 151, and 395 were immunized with a mixture of unconjugated peptides. Chimpanzee 395 was injected after two doses of 21 distinct V3 loop peptides with recombinant gp160²¹ and gag P18²¹ and with 100 μ g each of unconjugated V3 loop peptides from HIV-1 BRU, RF, MN, SF2, and ELI (dashed arrows). Chimpanzee 151 was boosted with 125–150 μ g of gp160²¹ on days 168, 186, and 326 (∇), after completion of the immunization schedule with V3 peptides. Chimpanzee 531 was immunized with free peptides followed by peptides linked to the nucleocapsid protein gag P18. Chimpanzees 529, 493, and 495 were immunized with peptides linked to recombinant *nef* protein. Chimpanzee 493 received together with the last dose of *nef*-peptide conjugates on day 181 also gp160.²¹ Chimpanzee 529 received with each dose of *nef*-peptide conjugates also gp160.²¹ All chimpanzees were immunized with peptides or peptide-carrier conjugates at day 0 followed by boosts with free peptides (indicated by arrows) or by

peptide carrier conjugates (indicated by arrowheads). Antibody responses to the synthetic peptides (\blacksquare) were measured at several time intervals during immunization. Responses to recombinant gp120 (\blacksquare) were measured at selected times at which the anti-peptide antibody response peaked. Antibody responses to carrier proteins P18 (\square) and *nef* (\square) were measured at the same time.

recombinant vaccinia virus VV-1139, that expresses a noncleavable version of gp160 BRU.²¹ Chimpanzees #495 and #529 were prevaccinated only with antigens unrelated to gp160: gag P18 and *vif* in the form of vaccinia virus recombinants.²¹ Chimpanzee #151 also received the latter two antigens as well as *nef*.

Prior to immunization with synthetic peptide immunogens, none of these chimpanzees had detectable IgG antibodies to the V3 hypervariable loop of gp160-III_B, closely related to the subtype of HIV-1 (LAV) used for preimmunization. All chimpanzees except #531 developed detectable IgG antibody responses to V3 hypervariable loop peptides only after the second injection of the peptide immunogens (Fig. 1). Subsequent boosts led to increased antibody levels to the loop peptides, with the exception of chimpanzee #495, a poor responder to the synthetic peptide components of the *nef* protein-peptide conjugate, although relatively high levels of anti-*nef* antibodies were detected in serum of this chimpanzee. The highest levels of anti-V3 loop peptide antibodies (endpoint titers $1: > 10^6$) were detected in sera of chimpanzees #499 and #529. The latter two chimpanzees were either preimmunized with gp160 (#499) or simultaneously vaccinated with gp160 and V3 loop peptides linked to *nef* (#529). Repeated boosting of chimpanzee #151 with gp160 after completion of the course of immunization did not result in increased antibody titers as measured by ELISA using either wells coated with a mixture of 21 distinct V3 peptides (Fig. 1) or wells coated with peptides from HIV-1 BH10, Z-3 and MAL, respectively (data not shown). Similarly, boosting of chimpanzee #395 with gp160 + gag P18 and with selected V3 peptides did not enhance the antibody response to the V3 loop. Cumulatively, these results suggest that either preimmunization with gp160 (gp120) or simultaneous administration of this recombinant protein(s) with V3 peptides elicits the highest antibody response to the principal neutralizing determinant. This conclusion is also confirmed by results of virus neutralization assays.

Serum specimens with the highest levels of anti-peptide antibodies were also assayed for antibodies recognizing recombinant gp120 and, if applicable, the carrier protein used. The highest levels of anti-gp120 antibodies (endpoint titer $1: > 5 \times 10^5$) were detected in sera of chimpanzees #499, #151, #395, and #529. Slightly lower levels of anti-gp120 antibodies were detected in serum of chimpanzee #493 (endpoint titer $1: 2.5 \times 10^5$). Thus, 5 out of 7 chimpanzees had high antibody responses to gp120 elicited either by unconjugated synthetic peptides or by peptides covalently linked to the carrier protein *nef*. Chimpanzee #531, immunized with unconjugated peptides followed by immunization with peptides linked to gag P18, developed relatively high levels of antibodies to the homologous peptides yet had low serum antibody levels against gp120. Chimpanzees #531 and #495 having the lowest antibody

responses to gp120 were, unlike all the other chimpanzees, not exposed to any immunogens containing gp160.

Earlier studies indicated that immunization of rabbits with a mixture of V3 loop peptides from 21 distinct HIV-1 isolates (clones) in either unconjugated form¹⁷ or in the form of conjugates with recombinant *nef* and *gag* P18 proteins, respectively,¹⁸ led to the production of antibodies recognizing each of the peptide components of the complex mixtures (endpoint titers: $1 > 10^4$). To determine whether or not immunization of chimpanzees with immunogens containing 21 distinct peptides led to antibody responses to each of the peptides, one of the serum specimens from chimpanzee #499 was tested for antibodies to each of the 21 peptides which were present in the immunogen used. Results presented in Figure 2 indicate that antibodies to each peptide were present in the serum sample collected 186 days after the first immunization with unconjugated peptides. The endpoint titers of antibodies recognizing the individual peptides were $1 > 10^4$, except those directed against V3 loop peptides from WMJ-2, LAV-MA, JY-1, Z-6, and ELI isolates (clones) of HIV-1, which all have a low immunological cross-reactivity with the IIIB V3 loop peptide.¹⁷ Thus, the antibody response appeared primarily directed against the IIIB peptide, related to the protein antigen used for primary immunization, and to other V3 loops more cross-reactive¹⁷ with this peptide.

Serum samples of all chimpanzees except #495 having low levels of anti-gp120 antibodies, were tested for antibodies neutralizing the IIIB strain of HIV-1. These tests were carried out with serum specimens collected at day 0 before immunization with the V3 hypervariable loop peptides, at the time corresponding to peak anti-V3 antibody levels after immunization with these peptides, and after additional boosting with recombinant gp160 (if applicable). The results of the tests are summarized in Figure 3. Sera from chimpanzees #151 and #529 preimmunized with recombinant vaccinia viruses expressing gp160 and antigens other than gp160, respectively, did not contain any detectable virus-neutralizing antibodies (VNAb). On the other hand, chimpanzees #395, #493, and #499, which were preimmunized with recombinant gp160, showed VNAb, the highest level of which was detected in serum of chimpanzee #499. After completion of the immunization schedule with 21

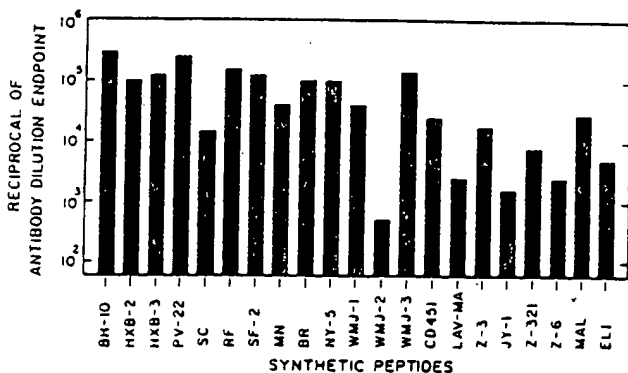


FIG. 2. Immune response to V3 hypervariable loop peptides from 21 distinct HIV-1 isolates of chimpanzee 499 immunized with a mixture of all 21 peptides simultaneously.

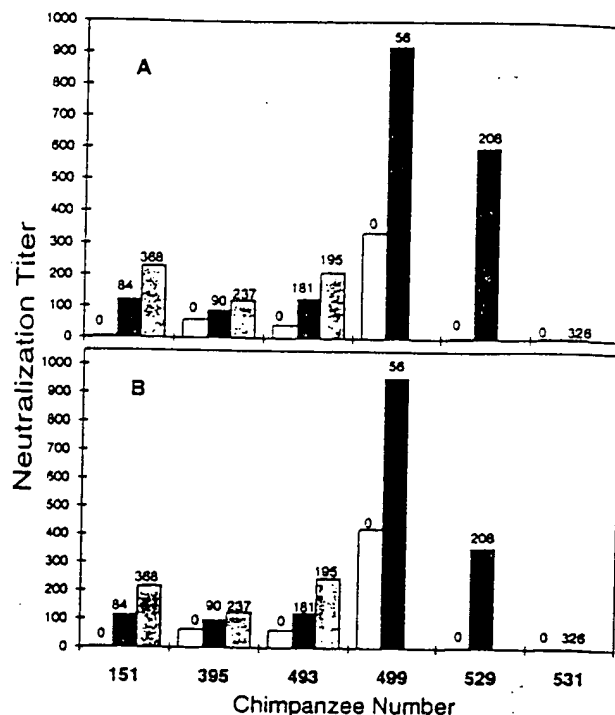


FIG. 3. Determination of virus-neutralizing antibodies (VNAb) in sera of chimpanzees immunized with preparations of V3 loop peptides from distinct HIV-1 isolates. VNAb were determined by inhibition of *gag* p24 synthesis (A) and by inhibition of HIV-1 cytopathic effects (B). VNAb were measured in serum specimens collected at day 0 before immunization with 21 distinct V3 hypervariable loop peptides (□), at the time of peak anti-V3 antibody levels after immunization with these peptides (■; numbers on top of the columns indicate days after the first immunization with V3 loop peptides) and after boosting with different preparations containing recombinant gp160 following completion of the immunization schedule with the 21 distinct V3 loop peptides (▨). For further details see FIG. 1.

hypervariable loop peptides, the level of VNAb increased in sera of all chimpanzees except #531, an animal which had not been preimmunized at all. These results suggest that VNAb were elicited by the peptides only in chimpanzees primed with gp160 either in the form of live recombinant vaccinia virus or of recombinant gp160. VNAb were present in sera of chimpanzees #395, #493, and #499 at a time when antibodies to the V3 hypervariable loop were not detectable by serological tests (see Fig. 1). Therefore, VNAb present in sera of these chimpanzees after completion of the immunization schedule with V3 peptides were expected to be directed against distinct epitopes on gp160. To explore this possibility, the serum of a selected chimpanzee (#499) collected 56 days after the first immunization with V3 hypervariable loop peptides was submitted to affinity chromatography on the V3-IIIB peptide linked to Sepharose. The unadsorbed fraction, which did not contain detectable antibodies to the V3-IIIB loop, and antibodies eluted from the column by 4 M $MgCl_2$, containing all of the original anti-V3 loop antibodies, were assayed for VNAb. VNAb were recovered in both the

column effluent (33%) and in eluate (67%). This is in agreement with the expectation that VN antibodies with distinct specificities were present in sera of the immunized chimpanzees, and that immunization with V3 loop peptides elicited V3-specific VNAb which were present at low or undetectable levels before immunization with the V3 loop peptides. After immunization with V3 peptides, the highest levels of VNAb were detected in sera of chimpanzees #499 and #529. These chimpanzees were either preimmunized with recombinant gp160 (#499) or simultaneously immunized with both V3 loop peptides (linked to *nef*) and gp160 (#529). Attempts to boost VNAb responses in chimpanzees #159, #395, and #493 by additional immunizations with gp160 after completion of the immunization schedule with V3 peptides led to a slight increase of the VNAb titer (≤ 2 -fold). Chimpanzee #499 having the highest level of serum VNAb was protected against challenge with HIV-1-IIIB.²¹ Cumulatively, these results suggest that only chimpanzees immunized with both gp160 and V3 hypervariable loop peptides, either consecutively or simultaneously, develop relatively high levels of VNAb specific for the V3 hypervariable loop.

The virus-neutralizing activities of serum from chimpanzee #499 against two additional HIV-1 isolates available in our laboratory, RF and MN, were also tested. The virus-neutralizing activity against the RF isolate was lower than that observed for the IIIB isolate and was borderline for the MN isolate (Fig. 4). Thus the virus-neutralizing activity against the RF isolate, which is less divergent from the IIIB isolate in the sequence of the V3 hypervariable loop in comparison with the MN isolate,¹⁷ was higher than that against the MN isolate, although the endpoint titer of antibodies determined in serological tests was higher for the MN than for the RF V3 loop (Fig. 2). Therefore, the virus-neutralizing activity of antisera cannot be predicted from results of immunoassays measuring antibodies to V3 loop peptides. In this respect, it should be mentioned that sera collected from chimpanzee #499 just before immunization with the peptides contained low levels of antibodies neutralizing the infectivity of the RF isolate (endpoint titer $\sim 1:70$).

Evidence suggesting that virus-neutralizing antibodies are directed against a preferred conformation of the V3 loop of HIV-1 gp120

Comparison of results presented in Figures 1 and 3 indicate a 10^4 – 10^5 -fold difference in magnitude of antibody titers determined by serological methods and by virus neutralization assays. This suggests the possibility that: (1) virus-neutralizing antibodies may represent only a subpopulation of antibodies recognizing epitopes on the V3 hypervariable loop of gp120, (2) epitopes on synthetic peptides corresponding to full-length V3 loops only partially mimic the corresponding loops in the native gp120 sequence, and (3) the conformation of the V3 loop within the envelope of HIV-1 may be affected by sequences which are adjacent to the V3 loop due to protein folding within the tertiary structure of gp120 but are not adjacent to this loop in the primary sequence of the glycoprotein. Experiments were carried out to investigate these possibilities.

The role of disulfide bonds between N- and C-terminal cysteine residues (303 and 338) of the full-length synthetic V3 loop peptide in antigenic mimicry of the corresponding loop in the gp120 glycoprotein was investigated. The oxidized V3

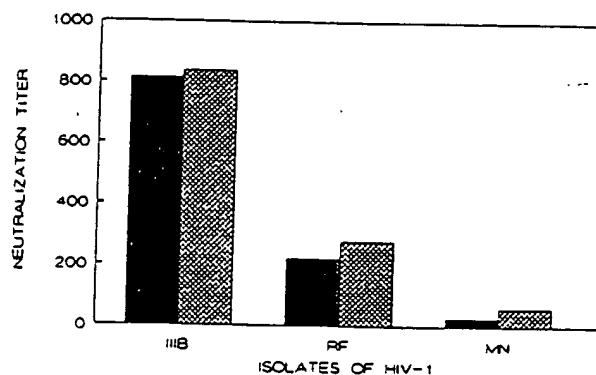


FIG. 4. Virus-neutralizing activity against distinct HIV-1 isolates of serum from chimpanzee #499 collected 186 days after primary immunization with unconjugated peptides corresponding to V3 loops of 21 distinct HIV-1 isolates (clones). Neutralizing antibodies were measured by inhibition of HIV-1 P24 antigen synthesis (▨) or by inhibition of the cytopathic effects HIV-1 (■). For further explanation, see Fig. 1.

peptide lacking free cysteine residues³² and the reduced and alkylated V3 peptide were tested as inhibitors of the reaction between peroxidase-labeled gp120 and monoclonal antibodies specific for the V3 loop. Results presented in Figure 5 show that reduction-alkylation decreased the inhibitory activity of the peptide ~ 19 - and 350-fold, respectively, for the reaction of gp120 with MAb 9305 and MAb 9284. This suggests that a synthetic V3 loop peptide resembles the corresponding loop in gp120 more than the "linear version" of the same peptide.

To be optimally effective against HIV-1 infection, immunization with synthetic V3 loop peptides should result in antibodies binding nearly equally well to V3 loops in the form of peptides or as domains in the gp120 structure. Antibodies elicited by the peptides should not contain predominant populations of immunoglobulins recognizing the peptides preferentially over the V3 loops of HIV-1. To determine whether or not rabbit anti-V3 peptide antibodies have such properties, the inhibitory effect of goat antibodies to gp120 on the reaction of anti-V3 antibodies with homologous peptides and with gp120, respectively, from two selected HIV-1 sequences, BH10 and SF2, was studied. Results presented in Figure 6 show that anti-gp120 antibodies efficiently inhibited the reaction of the anti-peptide antibodies with gp120 but were rather inefficient in inhibiting the attachment of anti-peptide antibodies to the homologous peptide antigens. This suggests that the binding constant for the reaction between rabbit anti-peptide antibodies and homologous peptides was higher than that corresponding to the reaction between anti-peptide antibodies and gp120. Alternatively, only a subpopulation of antibodies, elicited in rabbits by the peptide, recognized gp120, while the predominant antibody population was specific for the peptide only. The latter explanation is unlikely since the dilution endpoints of antibodies in sera of rabbits immunized with the V3 loop peptides were of similar orders of magnitude when measured by solid-phase immunoassays using either peptide- or gp120-coated wells.⁹ Results with chimpanzee antisera (Fig. 1) support this conclusion.

The discovery of neutralization escape mutants having V3 loop sequences identical to HIV-1 isolates which can be neutral-

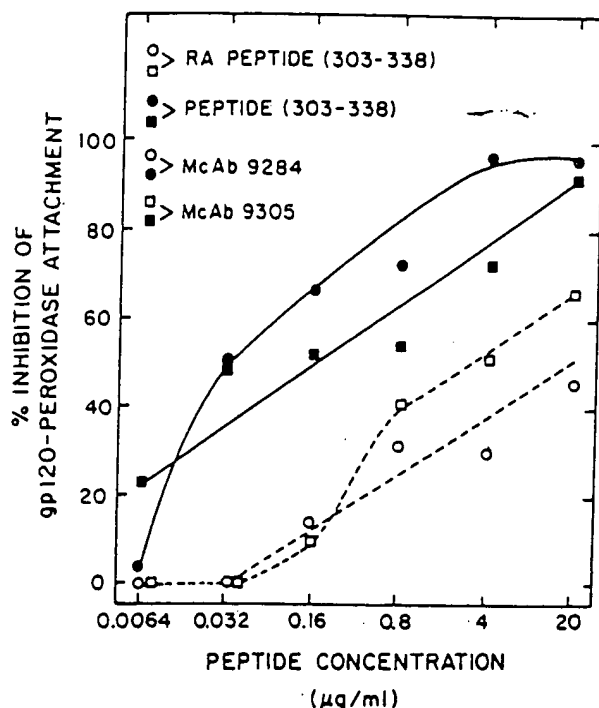


FIG. 5. Inhibitory effect of the V3 hypervariable loop peptide from gp120 of HIV-1 clone BH10 and the same peptide after reduction and alkylation (RA) on the attachment of horseradish peroxidase-labeled gp120 IIIB to monoclonal antibodies directed against the synthetic peptide RIQRGPGRFVTIGK, corresponding to residues 315-329 of the V3 hypervariable loop of HIV-1 clone BH10 (MAb 9305), and against the V3 hypervariable loop (raised by immunization with gp120 IIIB) (MAb 9284) each immobilized on wells of 96 well polystyrene plates.

ized indicates that amino acid replacements outside this loop may affect the recognition by antibodies of epitopes comprising the V3 loop.^{33,34} These results suggest that virus-neutralizing antibodies may be directed against conformation-dependent epitopes, only a portion of which correspond to sequences within the region (303-338) of gp120 IIIB. The following strategy for establishing the proximity to the V3 loop within the three-dimensional structure of gp120 of amino acid residues nonadjacent in the primary amino acid sequence was developed. The inhibitory effect of antisera specific for the V3 hypervariable loop (from species other than rabbits) on the reaction of distinct rabbit antipeptide sera (directed to segments of gp160) with gp160 was studied. It was expected that antibodies specific for the V3 loop would inhibit the attachment to gp160 of rabbit antibodies directed to those segments of gp160 which are adjacent to the V3 loop in the tertiary structure of the glycoprotein. MAb NEA9305, specific for a portion of the V3 loop, inhibited the attachment to gp160 of only 3 of 33 distinct antibodies to peptides from the sequence of gp160. These three antibodies were: anti-(303-338) and anti-(306-338), directed against the V3 hypervariable loop and anti-(113-142)_{AbA133} (Fig. 7). Goat antibodies to a synthetic peptide SP10 [= (303-328)] from the V3 hypervariable loop also selectively inhibited the attachment to gp160 of rabbit antisera against the

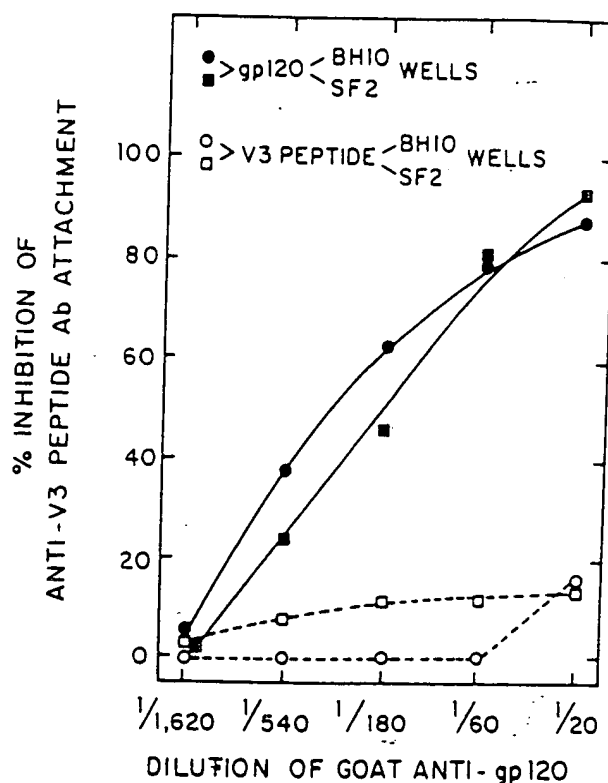


FIG. 6. Inhibitory effect of goat antibodies raised by immunization with gp120 of HIV-1, BH10, and SF2, respectively, on the reaction between rabbit antibodies, directed against BH10 and SF2 peptides from the V3 hypervariable loop of these HIV-1 isolates (clones), with wells coated with gp120 BH10 (●) and gp120 SF2 glycoproteins (■), respectively, and the corresponding V3 loop peptides (○, □).

V3 hypervariable loop [anti-(303-338) and anti-(306-338)] and against (113-142)_{AbA133} (Fig. 8). Since antibodies to the overlapping peptides (102-126) and (138-164), respectively, attached to gp160 to the same extent in the presence and absence of MAb NEA9305 (data not shown), residues within the sequence 127-137 are probably in the proximity of the V3 hypervariable loop. Since MAb NEA9305 did not inhibit the attachment of anti-(113-142)_{AbA126} to gp160, cysteine at position 126, but not at position 133, probably is important for this epitope and cannot be replaced by aminobutyric acid. These results agree with the observation that point mutations at position 135 affect the neutralizability of HIV-1 by antibodies.³³

Epitope mapping of antibodies from chimpanzees immunized with V3 hypervariable loop peptides

The chimpanzees in this study, except #531 and #495, were also immunized with antigens containing the gp120 sequence, either in the form of recombinant gp160 or of a vaccinia virus recombinant expressing gp160. Boosting with the synthetic peptides may have expanded a population of B cells resulting in the production of antibodies qualitatively distinct from those which would be produced by immunization with V3-loop peptides only. To investigate this possibility, antisera from

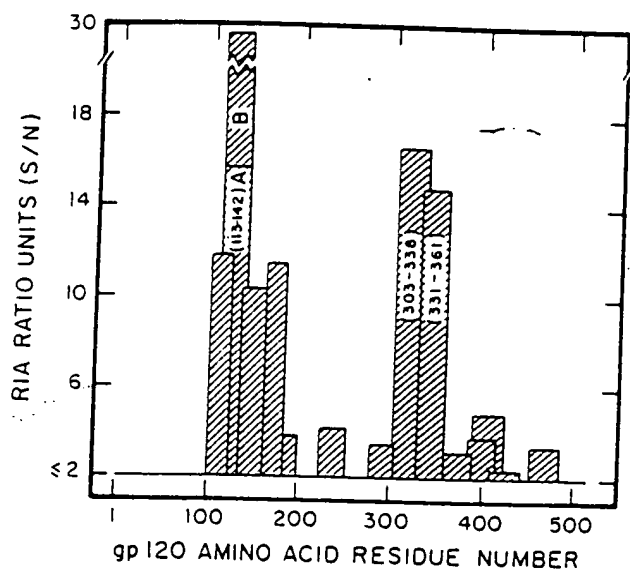


FIG. 9. Results of epitope mapping of antibodies in serum from chimpanzee #499 after completion of the immunization schedule with V3 hypervariable loop peptides. Peptide (113-142) was in two forms; A, with cysteine (Cys) at position 133 replaced by aminobutyric acid (Aba), and B, with Cys at position 126 replaced by Aba.

also had the highest levels of virus neutralizing antibodies (Fig. 3). These results indicate that only chimpanzees #499 and #529 developed high levels of high-affinity antibodies recognizing the V3 loop of native gp120.

DISCUSSION

It was reported that immunization with recombinant gp120 or gp160 elicited in humans and primates low levels of antibodies detectable by serological techniques while virus neutralizing antibodies were usually undetectable.²³⁻²⁵ Chimpanzees immunized with recombinant gp120 but not with gp160 developed antibodies reacting with the principal neutralizing determinant (PND) (maximum dilution endpoint 1:1,100) and virus-neutralizing antibodies (titer 1:320 to 1:640).²⁰ The chimpanzees immunized with recombinant gp120 were also protected against HIV-1 challenge virus. Results presented here indicate that chimpanzees preimmunized with preparations containing gp160 developed high levels of antibodies to the PND (as measured by ELISA tests with both homologous synthetic peptides and recombinant gp120 from HIV-1_{IIIIB}) only after boosting with synthetic V3 loop peptides. These results suggest that it is possible to elicit high levels of anti-PND antibodies by immunization of chimpanzees with unconjugated, full-length synthetic V3 loop peptides. However, the immune responses elicited by unconjugated V3 loop peptides or by loop peptides conjugated to HIV-1 carrier proteins *nef* and *gag* P18 were not uniform. Chimpanzee #495 vaccinated with V3 loop peptides linked to the *nef* protein responded very poorly to these peptides. Chimpanzee #531 immunized with the *gag* P18-peptide conjugate developed reasonable levels of antibodies to the peptides but low levels of antibodies recognizing gp120. The least successful

immunizations (chimpanzees #531 and #495) were observed with V3 loop peptides linked to carrier proteins. On the other hand, carrier-linked V3 peptides elicited in chimpanzees #493 and #529 high levels of antibodies recognizing the homologous peptides and gp120, suggesting that free and carrier-linked V3 peptides have similar immunogenicities in agreement with results obtained by immunization of rabbits.¹⁸ The inferior immune responses in chimpanzees #531 and #495 can rather be explained by the fact that they were the only animals in this study not also immunized with gp160 either in the form of a recombinant protein or a vaccinia virus expressing gp160.

Preimmunization with gp160 from HIV-1_{IIIIB} followed by boosting with a mixture of V3 loop peptides from 21 distinct HIV-1 isolates (clones) or simultaneous vaccination with gp160 and peptides apparently resulted in a preferential antibody response to V3 loops most closely related to the HIV-1 subtype of the immunogen (gp160) used for primary immunization (Figs. 2 and 4). Since PNDs from HIV-1 isolates restricted to certain geographical areas are relatively conserved and immunologically cross-reactive,^{14,15} it appears that it would be

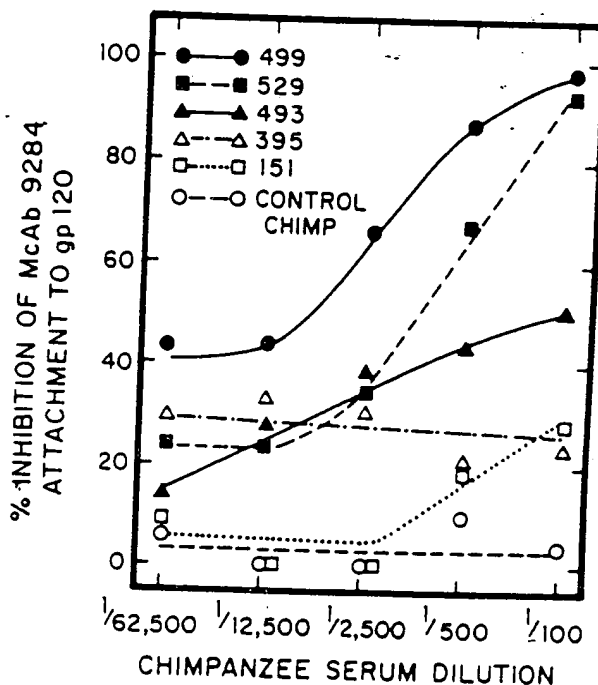


FIG. 10. Inhibitory effect of chimpanzee antisera on the attachment of mouse monoclonal antibodies raised by immunization with HIV-1_{IIIIB} gp120 and specific for the V3 loop (MAb NEA9284; 125 ng) to gp120. Serial dilution of each of the chimpanzee sera were assayed for inhibitory activity. Serum samples corresponding to the time of peak antibody responses (FIG. 1) were assayed. The same serum specimens were also tested for anti-gp120 and for anticarrier protein antibodies, if applicable, in addition to anti-peptide antibodies (FIG. 1). The optical density at 450 nm corresponding to MAb NEA9284 attached to gp120-coated wells in the absence of competing chimpanzee antibodies was 0.851. The optical density corresponding to controls in which normal mouse IgG was used instead of the MAb was 0.002.

preferable in future studies to use V3 loop peptide immunogens from fewer HIV-1 isolates and to possibly increase their dosages above those of the 21 distinct peptides used in the present study.

Results of comparative studies on antibodies elicited by V3 loop peptides and by gp120, respectively (Figs. 5, 6, 10), suggest that full-length V3 loop peptides do not mimic completely the V3 loops in native gp120. This may have resulted in the observed discordance between titers of antibodies as determined by ELISA tests and virus-neutralization assays. These findings suggest the need for development of other immunogens possibly involving shorter peptides from the V3 hypervariable loops,¹⁵ mimicking the PND with higher fidelity.

Epitope mapping of antibodies present in serum of chimpanzee #499, preimmunized with gp160 and boosted with V3 loop peptides, indicated that hyperimmunization led not only to increased generation of antibodies specific for the immunizing peptide, but also to production of antibodies directed against other segments of the gp120 sequence not adjacent to the V3 loop in the primary sequence, but apparently proximate to the V3 loop due to folding of the gp120 polypeptide (Figs. 7-9). The significance of this observation remains to be explained.

Recent published results demonstrate that repeated immunization with gp120 from a single HIV-1 isolate (clone) or with a mixture of different gp120s results primarily in the generation of noncross-reactive anti-gp120 antibodies. On the other hand, sequential immunization with gp120s from different HIV-1 strains elicited the production of highly cross-reactive antibodies.³⁶ It seems possible that sequential immunization with synthetic peptides encompassing the PND of distinct HIV-1 isolates may also be more efficient in eliciting broadly reactive virus-neutralizing antibodies in comparison with vaccination by a mixture of distinct V3 loop peptides employed in the present study. Additional investigations utilizing small animals, and later primates, would be needed to further pursue this possibility.

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REFERENCES

- Goudsmit J, Deboucq C, Melen RH, Smit L, Bakker M, Asher DM, Wolff AV, Gibbs Jr CJ, and Gajdusek DC: Human immunodeficiency type I neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. *Proc Natl Acad Sci (USA)* 1988;85:4478-4482.
- Matsushita S, Robert-Guroff M, Rusche J, Koito A, Hattori T, Hoshino H, Javaherian K, Takatsuki K, and Putney SD: Characterization of a human immunodeficiency virus neutralizing monoclonal antibody and mapping of the neutralizing epitope. *J Virol* 1988;62:2107-2114.
- Palker TJ, Clark ME, Langlois AJ, Matthews TJ, Weinhold KJ, Randall RR, Bolognesi DP, and Haynes BF: Type-specific neutralization of the human immunodeficiency virus with antibodies to env-encoded synthetic peptides. *Proc Natl Acad Sci (USA)* 1988;85:1932-1936.
- Rusche JR, Javaherian K, McDanal C, Petro J, Lynn DL, Grimala R, Langlois A, Galo RC, Arthur LO, Fischinger PJ, Bolognesi DP, Putney SD, and Matthews TJ: Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120. *Proc Natl Acad Sci (USA)* 1988;85:3198-3202.
- Linsley PS, Ledbetter JA, Kinney-Thomas E, and Hu SL: Effects of anti-gp120 monoclonal antibodies on CD4 receptor binding by the env protein of human immunodeficiency virus type 1. *J Virol* 1988;62:3695-3702.
- Skinner MA, Langlois AJ, McDanal CB, McDougal JS, Bolognesi DP, and Matthews TJ: Neutralizing antibodies to an immunodominant envelope sequence do not prevent gp120 binding to CD4. *J Virol* 1988;62:4195-4200.
- Javaherian K, Langlois AJ, McDanal C, Ross KL, Eckler LI, Jellis CL, Profy AT, Rusche JR, Bolognesi DP, Putney SD, and Matthews TJ: Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. *Proc Natl Acad Sci (USA)* 1989;86:6768-6772.
- Kenealy WR, Matthews TJ, Ganfield MC, Langlois AJ, Wasef-sky DM, and Petteway Jr SR: Antibodies from human immunodeficiency virus-infected individuals bind to a short amino acid sequence that elicits neutralizing antibodies in animals. *AIDS Res Human Retroviruses* 1989;5:173-182.
- Neurath AR, Strick N, and Lee EYS: B-cell epitope mapping of human immunodeficiency virus (HIV-1) envelope glycoprotein with long (19 to 36-residue) synthetic peptides. *J Gen Virol* 1990;71:85-95.
- Scott Jr. CF, Silver S, Profy AT, Putney SD, Langlois A, Weinhold K, and Robinson JE: Human monoclonal antibody that recognizes the V3 region of human immunodeficiency virus gp120 and neutralizes the human T-lymphotropic virus type III_{MAN} strain. *Proc Natl Acad Sci (USA)* 1990;87:8597-8601.
- Profy AT, Salinas PA, Eckler LI, Dunlop NM, Nara PL, and Putney SC: Epitopes recognized by the neutralizing antibodies of an HIV-1-infected individual. *J Immunol* 1990;144:4641-4647.
- Palker TJ, Matthews TJ, Langlois A, Tanner ME, Martin ME, Searce RM, Kim JE, Berzofsky JA, Bolognesi DP, and Haynes BF: Polyvalent human immunodeficiency virus synthetic immunogen comprised of envelope gp120 T helper cell sites and B cell neutralization epitopes. *J Immunol* 1989;142:3612-3619.
- Griffiths JC, Berrie EL, Holdsworth LN, Moore JP, Harris SJ, Senior JM, Kingsman SM, Kingsman AJ, and Adams SE: Induction of high-titer neutralizing antibodies, using hybrid human immunodeficiency virus V3-Ty viruslike particles in a clinically relevant adjuvant. *J Virol* 1991;65:450-456.
- LaRosa GJ, Davide JP, Weinhold K, Waterbury JA, Profy AT, Lewis JA, Langlois AJ, Dreesman GR, Boswell RN, Shaddock P, Holley LH, Karplus M, Bolognesi DP, Matthews TJ, Emini EA, and Putney SD: Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. *Science* 1990;249:932-935.
- Javaherian K, Langlois AJ, LaRosa GJ, Profy AT, Bolognesi DP,

- Herlihy WC, Putney SC, Matthews TJ: Broadly neutralizing antibodies elicited by the hypervariable neutralizing determinant of HIV-1. *Science* 1990;250:1590-1593.
16. Zwart G, Langeduk H, Van Der Hoek L, De Jong J-J, Wolf's TFW, Ramautarsing C, Bakker M, De Ronde A, and Goudsmit J: Immunodominance and antigenic variation of the principal neutralization domain of HIV-1. *Virology* 1991;181:481-489.
 17. Neurath AR and Strick N: Confronting the hypervariability of an immunodominant epitope eliciting virus neutralizing antibodies from the envelope glycoprotein of the human immunodeficiency virus type 1 (HIV-1). *Mol Immunol* 1990;27:539-549.
 18. Neurath AR, Strick N, Kolbe H, Kiény M-P, Girard M, and Jiang S: Confronting the hypervariability of an immunodominant epitope eliciting virus neutralizing antibodies from the envelope glycoprotein of the human immunodeficiency virus type 1 (HIV-1). II: Synthetic peptides linked to HIV-1 carrier proteins *gag* and *nef*. *Mol Immunol* 1991; in press.
 19. Emini EA, Nara PL, Schleif WA, Lewis JA, Davide JP, Lee DR, Kessler J, Conley S, Matsushita S, Putney SD, Gerety RJ, and Eichberg JW: Antibody-mediated in vitro neutralization of human immunodeficiency virus type 1 abolishes infectivity for chimpanzees. *J Virol* 1990;64:3674-3678.
 20. Berman PW, Gregory TJ, Riddle L, Nakamura GR, Champe MA, Porter JP, Wurm FM, Hershberg RD, Cobbs EK, and Eichberg JW: Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature* 1990;345:622-625.
 21. Girard M, Kiény M-P, Pinter A, Barre-Sinoussi F, Nara P, Kolbe H, Kusumi K, Chaput A, Reinhart T, Muchmore E, Ronco J, Kaczorek M, Gomard E, Gluckman J-C, and Fultz PN: Immunization of chimpanzees confers protection against challenge with human immunodeficiency virus. *Proc Natl Acad Sci (USA)* 1991;88:542-546.
 22. Arthur LO, Pyle SW, Nara PL, Bess Jr. JW, Gonda MA, Kelliher JC, Gilden RV, Robey G, Bolognesi DP, Gallo RC, and Fischinger PJ: Serological responses in chimpanzees inoculated with human immunodeficiency virus glycoprotein (gp120) subunit vaccine. *Proc Natl Acad Sci (USA)* 1987;84:8583-8587.
 23. Viscidi R, Ellerback E, Garrison L, Midthun K, Clements ML, Clayman B, Fernie B, Smith G, and NIAID Aids Vaccine Clinical Trials Network: Characterization of serum antibody responses to recombinant HIV-1 gp160 vaccine by enzyme immunoassay. *AIDS Res Human Retroviruses* 1990;6:1251-1256.
 24. Page M, Mills KHG, Schild GC, Ling C, Patel V, McKnight A, Barnard AL, Dilger P, and Thorpe R: Studies on the immunogenicity of Chinese hamster ovary cell-derived recombinant gp120 (HIV-1_{MS}). *Vaccine* 1991;9:47-52.
 25. Wintsh J, Chaignat C-L, Braun DG, Jeanneret M, Abrignani DG, Montagna D, Clavijo F, Moret P, Dayer J-M, Staehelin T, Doe B, Steimer KS, Dina D, and Cruchaud A: Safety and immunogenicity of a genetically engineered human immunodeficiency virus vaccine. *J Infect Dis* 1991;163:219-225.
 26. Hart MK, Palker TJ, Matthews TJ, Langlois AJ, Lerche NW, Martin ME, Searce RM, McDaniel C, Bolognesi DP, and Haynes BF: Synthetic peptides containing T and B cell epitopes from human immunodeficiency virus envelope gp120 induce anti-HIV proliferative responses and high titers of neutralizing antibodies in rhesus monkeys. *J Immunol* 1990;145:2677-2685.
 27. Takahashi H, Germain RN, Moss B, and Berzofsky JA: An immunodominant class I-restricted cytotoxic T lymphocyte determinant of human immunodeficiency virus type 1 induces CD4 class II-restricted help for itself. *J Exp Med* 1990;171:571-576.
 28. Takahashi H, Cohen J, Hosmalin A, Cease KB, Houghten R, Comette JL, Delisi C, Moss B, Germain RN, and Berzofsky JA: An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. *Proc Natl Acad Sci (USA)* 1988;85:3105-3109.
 29. Culmann B, Gomard E, Kiény M-P, Guy B, Dreyfus F, Saimot A-G, Sereni D, Sicard D, and Levy J-P: Six epitopes reacting with human cytotoxic CD8+ T cells in the central region of the HIV-1 NEF protein. *J Immunol* 1991;146:1560-1565.
 30. Allison AC and Byars NE: An adjuvant formulation that selectively elicits the formation of antibodies of protective isotypes and of cell-mediated immunity. *J Immunol Methods* 1986;95:157-168.
 31. Ritchie DG, Nickerson JM, and Fuller GM: Two simple programs for the analysis of data from enzyme-linked immuno-sorbent assays (ELISA) on a programmable desk-top calculator. *Meth Enzymol* 1983;92:577-588.
 32. Neurath AR, Strick N, Fields R, and Jiang S: Peptides mimicking disulfide loops in HIV-1 gp120, other than V3, do not elicit virus neutralizing antibodies. *AIDS Res Human Retroviruses* 1991; 7:657-662.
 33. Willey, RL, Ross EK, Buckler-White AJ, Theodore TS, and Martin MA: Functional interaction of constant and variable domains of human immunodeficiency virus type 1 gp120. *J Virol* 1989; 63:3595-3600.
 34. Nara PL, Smit L, Dunlop N, Hatch W, Merges M, Waters D, Kelliher J, Gallo RC, Fischinger PJ, and Goudsmit J: Emergence of viruses resistant to neutralization by V3-specific antibodies in experimental human immunodeficiency virus type 1 IIIB infection of chimpanzees. *J Virol* 1990;64:3779-3791.
 35. Neurath AR, Strick N, and Jiang S: Recognition of the V3 hypervariable loop of HIV-1 gp120 is not determined exclusively by its primary amino acid sequence. In: *Vaccines 91: Modern Approaches to New Vaccines Including Prevention of AIDS*. RM Chanock, HS Ginsberg, F Brown and RA Lerner (eds.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1991, pp. 15-21.
 36. Klinman DM, Higgins KW, and Conover J: Sequential immunizations with rgp120s from independent isolates of human immunodeficiency virus type 1 induce the preferential expansion of broadly crossreactive B cells. *J Exp Med* 1991;173:881-887.

Address reprint requests to:

A. Robert Neurath
Senior Investigator
Biochemical Virology Laboratory
The New York Blood Center
310 East 67th Street
New York, NY 10021